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Expression of chicken parvovirus VP2 in chicken embryo fibroblasts requires codon optimization for production of naked DNA and vectored meleagrid herpesvirus type 1 vaccines

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Abstract Meleagrid herpesvirus type 1 (MeHV-1) is an ideal vector for the expression of antigens from pathogenic avian organisms in order to generate vaccines. Chicken parvovirus (ChPV) is a widespread infectious virus that causes serious disease in chickens. It is one of the etiological agents largely suspected in causing Runting Stunting Syndrome (RSS) in chickens. Initial attempts to express the wild-type gene encoding the capsid protein VP2 of ChPV by insertion into the thymidine kinase gene of MeHV-1 were unsuccessful. However, transient expression of a codon-optimized synthetic VP2 gene cloned into the bicistronic vector pIRES2-Ds-Red2, could be demonstrated by immunocytochemical staining of transfected chicken embryo fibroblasts (CEFs). Red fluorescence could also be detected in these transfected cells since the red fluorescent protein gene is downstream from the internal ribosome entry site (IRES). Strikingly, fluorescence could not be demonstrated in cells transiently transfected with the bicistronic vector containing the wild-type or non-codon-optimized VP2 gene. Immunocytochemical staining of these cells also failed to demonstrate expression of wild-type VP2, indicating that the lack of expression was at the RNA level and the VP2 protein was not toxic to CEFs. Chickens vaccinated with a DNA vaccine consisting of the bicistronic vector containing the codon-optimized VP2 elicited a humoral immune response as measured by a VP2-specific ELISA. This VP2 codon-optimized bicistronic cassette was rescued into the MeHV-1 genome generating a vectored vaccine against ChPV disease.

Keywords Chicken parvovirus · VP2 · Codon-optimized · Bicistronic vector · DNA vaccine · Meleagrid herpesvirus vector

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Introduction

Parvoviruses are members of the small non-enveloped DNA containing viruses in the family Parvoviridae that cause multiple diseases in animals and humans. Since the virus requires actively dividing cells in order to replicate, cells in the gastrointestinal tract are often the site of virus replication [1]. Young animals are especially vulnerable to parvoviral infection with symptoms of vomiting, diarrhea, and immuno-suppression [2]. The most notable and costly animal diseases associated with parvoviruses are those caused by porcine, feline, and canine parvoviruses with mortality rates as high as 80 % in young puppies [3]. In humans, the parvovirus B19 causes Erythema infectiosum (Fifth disease) in children [4]. Parvovirus infections in the avian species geese, chickens, and ducks have also been described [5, 6]. Studies conducted in the 1980s have identified parvoviruses in the guts of chickens with symptoms of emaciation and enteropathy [7–9]. In experimentally infected 1-day-old specific pathogen free (SPF) chickens, ChPV has been demonstrated to cause the occurrence of clinical symptom resembling Runting Stunting Syndrome (RSS) [10–12].

Currently, the evidence suggests that ChPV is one of the etiological agents responsible for RSS, also described as malabsorption syndrome and rarely as the pale bird syndrome or brittle bone disease [13, 14]. In young birds, the clinical symptoms are noticeable as runting, diarrhea with

subsequent increase in morbidity and mortality. In the U.S. during the last few years the occurrence of RSS has been observed mainly in broiler chickens. Using a PCR assay that targeted the NS1 gene, Zsak et al. [15] found parvovirus infections in 77 % of broiler flock samples from 54 farms and 78 % of turkey samples from 29 farms. In Europe, a Polish study demonstrated that 22.2 % of 48 chicken flocks were positive for ChPV infections using a similar PCR-based method [16].

Because of its economic importance in the poultry industry, vaccines against chicken parvovirus (ChPV) are needed to help curtail RSS. Structurally, ChPV virions are assembled from 32 capsomeres in an icosahedral symmetry of ~20 nm in diameter. The naked capsids enclose a genome consisting of single-stranded, negative-sense 4–6 Kb DNA which encodes three ORFs. The 3′ most ORF encodes the structural capsomere proteins (VP1 and VP2) which are the major antigenic determinants. VP1 and VP2 are spliced variants and it is believed that VP2 is the major structural protein comprising the ChPV virions. Because of this, expression of VP2 as a naked DNA vaccine or vectored into the meleagrid herpesvirus genome could prove useful in vaccine development. In order to achieve this, it was necessary and essential to optimize the codons in the VP2 ORF of ChPV for expression in chicken embryo fibroblasts (CEFs), cells commonly used in vaccine development. Although this need was somewhat surprising, codon optimization for expression of VP2 for other parvoviruses has been reported [17, 18]. In particular, Zhi et al. have reported that transient expression of VP2 of the human parvovirus B19 in transfected non-permissive HeLa cells absolutely required codon optimization. Expression of non-optimized VP2 could not be detected in non-permissive HeLA cells although various levels of expression could be demonstrated in other permissive cell lines [19].

Materials and methods

Meleagrid herpesvirus type 1 (MeHV-1) strain FC-126 at passage 25 was used in this study. Primary or secondary CEFs were maintained in Dulbecco's modified Eagle medium supplemented with 5 % fetal bovine serum (FBS) and antibiotics. Nucleocapsid DNA was isolated from infected CEFs using the methods described by Volkening and Spatz [20].

Construction of donor plasmid containing wild-type VP2 (pL14)

The mammalian and prokaryotic expression vector pBK-CMV-lacsmGFP (Stratagene, La Jolla, CA) was modified to contain the additional restriction endonuclease sites *Ase*I, *Asc*I, and *Bgl*II. To accomplish this, two oligonucleotides were synthesized (ATTCATATGGATTAATGTC ATGATAATAATGG and AAGCATATGGCGCGCCA CTAGATCTGACAGTTACCAATGCTTAATC) and used in an amplification reaction with pUC19 as template resulting in the production of a 1,067 bp fragment containing the ampicillin gene. The fragment was digested with *Nde*I and cloned into *Ase*I-digested pBK-CMV-lacsmGFP to generate pAMP-3. This construct was further modified to contain meleagrid herpesvirus 1 sequences (i.e., flanks) for insertion into the thymidine kinase gene. To accomplish this, two PCR products were generated using the primer pair ATGATTAATGCGAGCTTGAAGATGATG and TAGA ACATTACAGAAGATATCCAGGTTATTGGACGTTAGT AGC and the primer pair ACGTCCAATAACCTGGATAT CTTCTGTAATGTTCTACCAAC and ATTAGATCTCTA CATTTAGATGATAACCGATCG with MeHV-1 DNA as a template, resulting in 588 and 552 bp PCR products, respectively. These products were then used as templates to generate a 1,104 bp fusion PCR product using the outside primers. The fusion PCR product was then digested with *Ase*I and *Bgl*II and cloned into *Ase*I/*Bgl*II digested pAMP-3 to generate pEF3. Plasmid pEF3 was modified to contain an ampicillin gene between the MeHV-1 right and left thymidine kinase flanks by amplifying the ampicillin gene of pUC19 with primer pair CTGGATATCTTAATGTCATGATAATAATGG and ATC AAGCTTACCAATGCTTAATCAGTGA. The resulting 1,041 bp fragment was digested with *Eco*RV and *Hind*III and cloned into a partially *Hind*III-digested pEF3. The resulting plasmid called pB2-8 was then isolated from an ampicillin resistant green fluorescent colony for subsequent cloning of the ChPV.

The VP2 gene was amplified using primers TGAGC TAGCCGCCATGATGGCAGATGAAAATGAACC and AAACTCGAGTTAGTTGGTCCGCGGCGCGCGCTTGG and pVP2 Baculovirus (BV) [21] as a template to generate a 1,633 bp fragment which was subsequently cloned into topo vector pCR2.1 (Invitrogen, Carlsbad, CA) to generate p7C-18. The sequence of this recombinant was then determined to ensure no detrimental mutations were introduced during PCR amplification. The VP2 gene was excised from p7C-18 following digestion with *NheI* and *Xho*I and cloned into *Nhe*I/*Xho*I-digested pB2-8 to generate pL14.

Generation of MeHV-1 bacterial artificial chromosome recombinants containing ChPV VP2 and smGFP

Both plasmid pB2-8 containing the GFP gene and pL14 containing the ChPV VP2 gene were linearized with *Hin-d*III and *Eco*RV, respectively, and then individually transformed into a competent *E. coli* strain containing a MeHV-1 bacterial artificial chromosome (BAC) (a gift from

Venugopal Nair, Institute for Animal Health, UK) [22] and recombinase plasmid pRedET (Gene Bridges, Heidelberg, Germany) in order to transfer these genes into the TK locus within the meleagrid herpesvirus genome. Recombinants were selected on LB plates containing chloramphenicol (12.5 μg/ml) and kanamycin (25 μg/ml). Integration was confirmed using an internal primer (CGGCAGGCCCT GCCATAGCC) specific for VP2 plasmid sequences and another primer (TGTATGATTGGCGTATGAACC) specific for the meleagrid genomic sequences outside those used for homologous recombination.

Cloning of codon-optimized ChPV VP2 into pIRES2-DsRed2

The codons for the VP2 ORF were optimized for the translational machinery of *Gallus gallus domesticus* using proprietary software (Genscript, Piscataway, NJ). The codon-optimized VP2 ORF was chemically synthesized (Genscript, Piscataway, NJ) to contain *Nhe*I and *XhoI* restriction endonuclease sites at the 5′ and 3′ termini of the ORF and cloned into a proprietary vector pUC57 (Fig. 1). The synthetic codon-optimized VP2 ORF was excised from the vector following *Nhe*I and *Xho*I digestion and ligated into *Nhe*I/*Xho*I-digested bicistronic internal ribosome entry site (IRES) vector pIRES2-DsRed2 (Clontech, Mountain-View, CA) with T4 DNA ligase to yield pSynVP2-DsRed2. Likewise the wild-type VP2 ORF was cloned into pIRES2-DsRed2 using a PCR/cloning scheme that involved oligonucleotides TCCGCTAGCAGAATTAGAAGAAATGAT GG and GAGCTCGAGAGTTACATTAGTTGG and template pL14 along with Platinum Taq polymerase (Life Technologies, Carlsbad, CA). This generated a recombinant called pNonSynVP2-DsRed2.

Plasmid transfection of DF-1 cells

The CEF immortalized cell line DF-1 [23] were grown to 80 % confluency in Dulbecco's modified Eagle media supplemented with 5 % FBS and antibiotics in 12-well dishes at 37 °C in a 5 % CO_2 incubator for 24 h. For transfection, 1.5 μl of X-tremeGENE 9 DNA transfection reagent (Roche, Indianapolis, IN) was added to 50 μl of serum free media (Opti-MEM, Invitrogen, Carlsbad, CA), in a micro-centrifuge tube and 1.0 μg of plasmid DNA either pSynVP2-DsRed2 or pNonSynVP2-DsRed2 was added to form the lipid–DNA complexes for 15 min at room temperature. The media in the 12-well dishes was replaced with 1.0 ml DMEM containing 5 % FBS and the transfection mixture was added to the attached cells. After incubating at 37 °C for 48 h the VP2 protein expressed in DF-1 cells was detected by an indirect immunohistochemical assay. At the same time, the expression of RFP was monitored using fluorescence microscopy.

Production of anti-peptide ChPV VP2 sera

The antigenic structure of chicken VP2 was analyzed for epitopes using the BepiPred linear epitope prediction method [24] through the web-based antigen profiler program (http://tools.immuneepitope.org/tools/bcell/iedb_input). A major antigenic region was identified between residues 247 and 356. Specific antisera was generated commercially (Open Biosystems, Rockford, IL) in rabbits with the synthetic peptides QRQNPLYDTWNVNGRGDD and PGMPQYRSESDKDEYLHT conjugated to KLH using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester as the divalent linking agent. Rabbit immunizations were carried out by intramuscular injection of 500 μg of antigen on day 1 (complete Freund's adjuvant) and boost on days 14, 28, and 42 (incomplete Freund's adjuvant) with 250 μg of antigen. Blood samples were collected at days 0 and 35 and days 56 and 58 after the third boost. Antisera from the two rabbits were checked for anti-peptide antibodies by indirect enzyme-linked immunosorbent assay (ELISA) using the corresponding peptide as antigen.

Immunocytochemical staining

DF-1 cells transfected with the pIRES constructs (after 48 h) were fixed and 4 % paraformaldehyde for 10 min at room temperature and permeabilized with 0.2 % Triton X 100 for 5 min at room temperature. CEFs transfected with recombinant MeHV-1 (after 96 h) were fixed with solutions containing 50 % methanol/50 % acetone. Non-specific protein binding sites were blocked with 10 % normal goat serum in 1× phosphate buffered saline (PBS). Cells were then incubated with rabbit anti-ChPV VP2 sera at a 1:200 dilution. After an overnight incubation at 4 °C and washings, the DF-1 cells were incubated with a fluorescein anti-rabbit IgG conjugate (7.5 μg/ml) and the recombinant MeHV-1 infected CEFs were incubated with an alkaline phosphatase mouse anti-rabbit IgG conjugate for 60 min at room temperature. After washing, visualization of expression of RFP and VP2 was demonstrated in transfected DF-1 cells using fluorescence microscopy with two different filters. In CEFs infected with recombinant MeHV-1, expression of VP2 was demonstrated following the addition (after 15 min) of the substrate Fast Red (Biogenix Fremont CA).

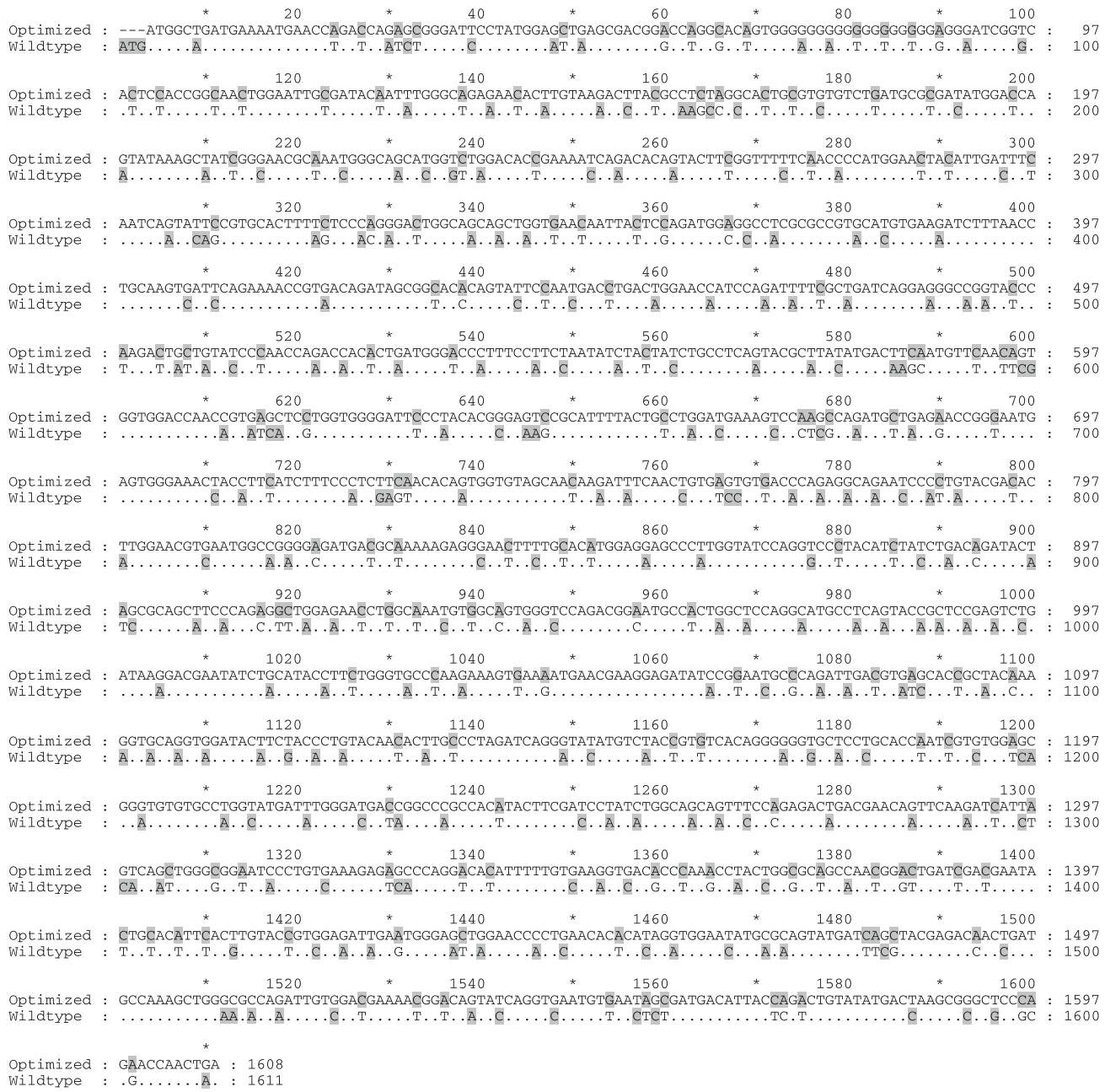

Fig. 1 Alignment of codon-optimized VP2 and original wild-type sequences

DNA immunization of chickens with chicken parvovirus VP2 gene

SPF white rock chickens were obtained from the Southeast Poultry Research Laboratory (SEPRL) flocks at 1 day of age and divided into three groups, with 10 birds in one group and five birds in each of two other groups. Among other agents, these flocks are monitored for avian reovirus, astrovirus, adenovirus, infectious bursal disease virus, and chicken anaemia virus [25]. Chickens were housed in Horsfal isolators (Federal Designs, Inc., Comer, G A) with ad libitum access to feed and water. General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [26]. At the age of 2 days, a group of chickens ($n = 10$) was terminally bled to measure maternally acquired serum antibodies to ChPV. At the same time, one group of chickens ($n = 5$) was inoculated intramuscularly with pSynVP2-DsRed2 (5 μg per chicken) in the presence

of 10 % Montanide gel adjuvant (SEPPIC) using a 12 mm long 20-gage sterile needle. The other group of chickens ($n = 5$) was inoculated with a mixture of PBS and Montanide adjuvant as control. Two weeks later, all of the VP2 immunized and control chickens were bled, and boost-inoculated with either 5 μg of pSynVP2-DsRed2/Montanide adjuvant or PBS/Montanide adjuvant, respectively. The experiment was terminated 2 weeks post-boost inoculation and the birds were bled and euthanized humanely. All inoculated and control chickens were bled from the ulnar (wing) vein using microtainer serum collectors (Becton–Dickinson, Franklin Lakes, NJ), serum was centrifuged at 3,000×*g* for 15 min and the supernatants were kept at −20 °C until tested.

ELISA

ChPV-specific serum antibodies were assayed using an ELISA as described previously [21]. Briefly, ChPV VP2 protein was expressed in a BV recombinant and purified VP2 was then used in capture ELISA. In the assay, 96-well white, flat-bottom Nunc plates (Fisher Scientific, Pittsburgh, PA) were coated with 500 ng of BV-VP2 infected SF9 cell lysates (48 wells on the left side of the plates) and 500 ng of parental BV (BVAg) infected SF9 cell lysates (48 wells on the right side of the plates). Coating, washing, blocking, and diluting reagents were purchased from Immunochemistry Technologies (Bloomington, MN). Test sera from chickens were used at 1:200 dilutions and secondary anti-chicken antibodies (GenWay Biotech, San Diego, CA) were applied at a 1:1000 dilution. Bound secondary antibody was measured by chemiluminescence after a 2 min incubation with 100 ml solution of LumiGLO, a luminol-based chemiluminescent substrate (KPL Inc., Gaithersburg, MD). Plates were read on a Biotek FL 3 800 microtiter plate luminometer (Biotek Instruments, Winooski, VT) with a 0.2 s read time per well. To determine the correlation between luminescence reading values and concentration of serum antibodies to ChPV VP2, the relative light unit (RLU) obtained for test serum was divided by RLU values for BVAg (negative antigen). Test sera were considered positive to ChPV VP2 when they had an RLU ratio of two or higher as described previously [21].

Statistical analysis

Data obtained from ELISA were statistically analyzed using the Student *t* test (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html). Experimental group means were considered significantly different from each other if $p < 0.05$.

Generation of an MeHV-1 recombinant expressing VP2 IRES2-DsRed2 bicistronic mRNA

The bicistron-containing codon-optimized VP2-IRES-RFP cassette and regulatory elements were transferred into a donor plasmid containing flanking sequences necessary for homologous recombination in the thymidine kinase locus within the MeHV-1 genome. To accomplish this, a large synthetic oligonucleotide of 394 bp containing the right and left flanking sequences of the thymidine kinase gene of MeHV-1 [map coordinates 46,768–46,935 and 47,383–47,552 of the MeHV-1 genome (accession number NC_002641)] was synthesized (IDT DNA, Coralville, IA) and cloned into proprietary vector IDT pSMART KAN to generate pMiniHVT-TK-SMART. PCR primers CACGGC GCGCCTAGTTATTAATAGTAATCAATTACG and TA TGGCGCGCCCACTCAACCCTATCTCGGTC along with Platinum Taq polymerase were used to amplify a 3,942 bp product using pSynVP2-DsRed2 as template. PCR product and pMiniHVT-TK-SMART were both digested with *Asc*I and ligated together using T4 DNA ligase. The orientation of the bicistron was confirmed with restriction endonuclease digestions of isolated plasmid DNA (Qiagen, Valencia, CA). One microgram of plasmids with either orientation of the bicistron (named VP2-10 and VP2-4) linearized with *Nru*I digestion and ten micrograms of MeHV-1 DNA was used in a calcium phosphate co-transfection of CEFs according to the methodology described by Morgan et al. [27]. Meleagrid herpesvirus recombinants expressing codon-optimized VP2 were plaque purified three times in CEFs.

Results and discussion

Lack of expression of ChPV VP2 from a recombinant Meleagrid herpesvirus bacterial artificial chromosome following CEF transfection

Expression of foreign genes encoding antigens for avian pathogens from the genome of the alphaherpesvirus meleagrid herpesvirus type I has been very successful in generating vectored vaccines. Here, we attempted to express the VP2 gene of ChPV in such a vectored system by cloning the nucleotide sequence of the VP2 gene derived from the virus into a donor vector containing flanking thymidine kinase sequences of MeHV-1 and rescuing it into a MeHV-1 BAC. Unfortunately, no expression could be demonstrated using immunocytochemical staining, although integration of the VP2 construct (containing wild-type VP2) into the MeHV-1 BAC could be confirmed using PCR with primers specific for the VP2 gene and MeHV-1 thymidine kinase sequences downstream from

those used for homologous recombination. A correct PCR product of 674 bp was generated in a reaction containing the recombinant VP2/MeHV-1 BAC (data not shown). Transient transfection experiments in CEFs using only the donor plasmid containing the wild-type VP2 gene were also unsuccessful in demonstrating expression. However, using a similar strategy in which the VP2 gene in the donor construct was swapped with green fluorescent protein gene (smGFP), efficient expression could be demonstrated upon transfection of the rescued recombinant MeHV-1 BAC expressing green fluorescent protein. Attempts to explain this lack of expression first involved nucleotide sequence determination of the whole donor plasmid, which proved to be correct, and subsequently fusion of an HA epitope tag onto the carboxy termini of wild-type VP2. Transient transfection of CEF's with this construct also failed to generate a signal upon immunocytochemical staining with HA-specific sera.

Expression of chicken parvovirus VP2 requires codon optimization

To determine whether the VP2 protein of ChPV was toxic to CEFs, other cell lines (i.e., Vero, LMH, DF-1) were used in transient transfection experiments with donor plasmid containing wild-type VP2. These experiments also failed to demonstrate expression. Because of this, we investigated whether the lack of expression was at the level of translation by generating a synthetic VP2 construct containing codons-optimized for expression (Fig. 1) based on the codon usage frequency of ORFs in the *Gallus gallus domesticus* genome. This synthetic codon-optimized gene was cloned upstream of an IRES in the bicistronic vector pIRES2-DsRed2 as shown in Fig. 2. Following transfection into DF-1 cells this construct not only fluoresced red (Fig. 3a) but upon indirect immunocytochemical staining with rabbit anti-chicken VP2 and fluorescein anti-rabbit IgG conjugate, a strong signal was detected (Fig. 3b). Interestingly, when this transfection experiment was done with the bicistronic construct containing wild-type VP2 sequence (non-codon-optimized) downstream of the same promoter used in the synthetic codon-optimized construct, neither fluorescence nor expression of the VP2 protein could be demonstrated (Fig. 3d). A bicistronic construct containing wild-type VP2 with a premature stop codon (TAA) after the codon for Leu264 was also constructed and failed to express both VP2 and RFP (data not shown). It is noteworthy that although RFP expressing cells could also be stained for VP2 expression, some transfected cells only expressed VP2 (Fig. 3c). Lack of expression of the downstream RFP gene is likely the result of inefficient cap-independent translation from the IRES. It has been reported that under the best of circumstances cap-independent initiation is only 25 % as efficient as cap-dependent initiation [28]. Since the codon-optimized transfection experiment clearly demonstrated that the ChPV VP2 protein was not

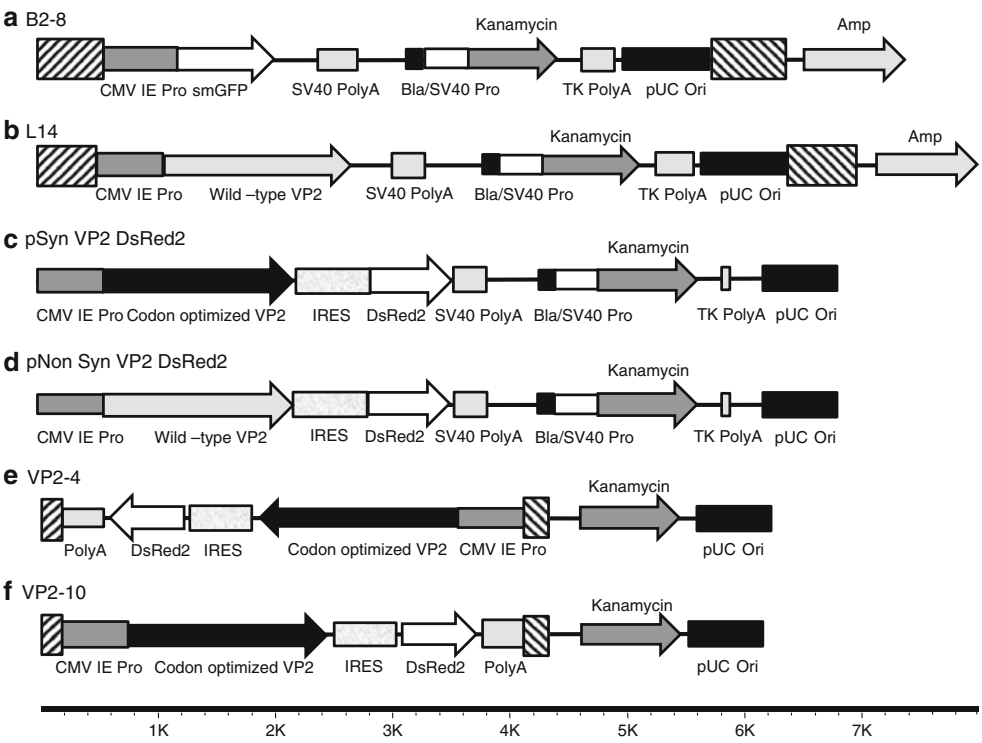

Fig. 2 The cloning strategy for construction of pSynVP2-DsRed2

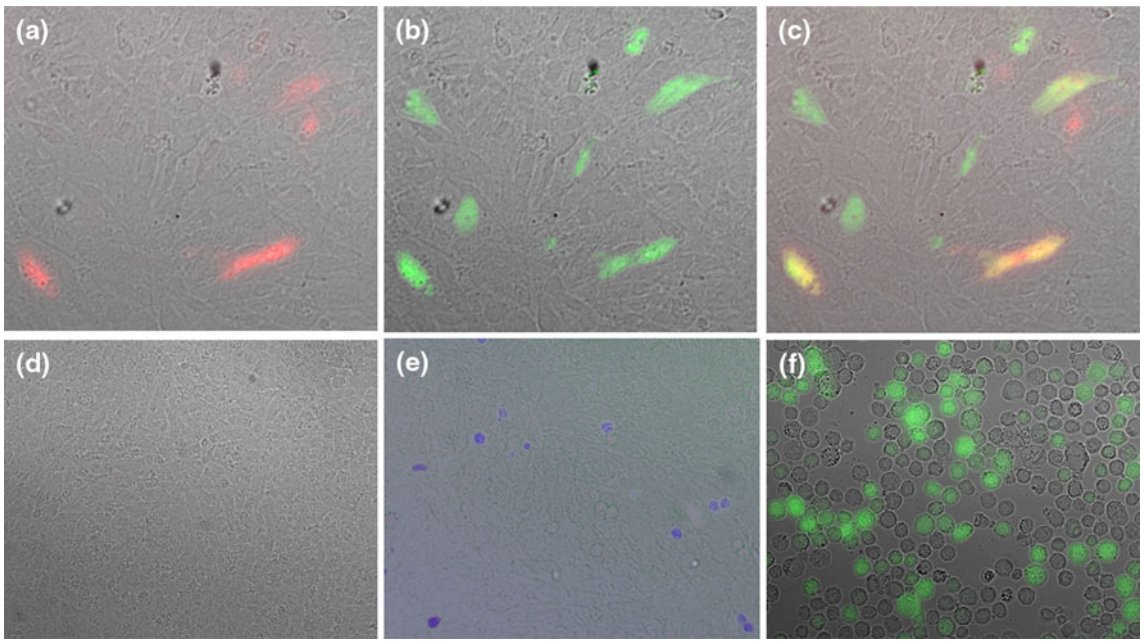

Fig. 3 Expression of the codon-optimized VP2 of chicken parvovirus in the immortal CEF cell line DF-1. Cells were transfected with plasmids pSynVP2-DsRed2, pNon SynVP2-DsRed2, and pLifeACT-mTurquoise2 (University of Amsterdam, NL) using X-tremeGENE. RFP expression was detected by fluorescence microscopy only in cells transfected with codon-optimized construct **a** pSynVP2-DsRed2, **b** pSynVP2-DsRed2 transfected cells expressing VP2 as detected by indirect immunofluorescence assay (IFA) with a fluorescein anti-rabbit IgG conjugate **c** merged image showing cells express both RFP and VP2. **d** DF-1 cells transfected with pNon SynVP2-DsRed2 containing wild-type VP2 sequence. **e** DF-1 cells transfected with pLifeACT-mTurquoise2 (transfection control). **f** IFA of SF9 cells infected with recombinant baculovirus expressing chick parvovirus VP2 (IFA control)

toxic to CEFs, the lack of expression of both wild-type VP2 and RFP from the bicistronic mRNA was due to the nucleotide sequence of wild-type VP2 and not its translation product. An analysis of the wild-type VP2 mRNA secondary structure using the web-based UNAfold program [29] (http://mfold.rna.albany.edu) identified three areas containing palindrome sequences that could form hairpin structures similar to those of microRNAs. These localized to positions 249–272 (AAACCAAACACAATACTTTG GTTT), 462–509 (AACAATCCAAATATTTGCAGATCA GGAAGGCAGATATCCTAGATTGTT), and 1081–1118 (GATGTATCCACTGCAACCAAAGTACAAGTAGATA CATC) on the VP2 gene with hairpin dG values of −5.30, −11.40, and −11.10, respectively. Palindrome sequences with comparable dG values were largely absent from the folded secondary structure generated with codon-optimized VP2. A search of chicken sequences in GenBank has identified two regions on chromosome Z within the chicken genome containing significant homology to two of the three identified hairpin structures in the WT VP2 mRNA: hairpin 249–272 [Identities = 21/22 (95 %), Gaps = 0/22 (0 %)] and hairpin 1081–1118 [Identities = 18/18 (100 %), Gaps = 0/18 (0 %)]. Although it is tempting to speculate that these represent targets for microRNA inhibition, further work is needed to experimentally investigate this hypothesis.

DNA vaccination of chickens with codon-optimized VP2

After characterizing the expression in vitro, the bicistronic plasmid containing the codon-optimized VP2 gene was injected into chickens in order to assess its value as a DNA vaccine. Prior to vaccination, 10 randomly chosen chickens (2 days of age) were terminally bled to confirm the lack of maternally acquired ChPV-specific antibodies. The blood from each chicken was withdrawn at 2 weeks post-first vaccination and the humoral antibody response was determined by an ELISA developed by Strother and Zsak [21]. None of the inoculated chickens developed ChPV VP2-specific antibody response following the first immunization ($p = 0.032$) and there was no specific antibody response in control chickens receiving just the adjuvant (Fig. 4). However, four of five VP2 immunized chickens in the booster group which received an additional dose of bicistronic vector on day 14 developed a positive immune response ($p < 0.01$) against ChPV VP2 indicating an anamnestic response. It is noteworthy that 60 % of the birds at day 28 in the booster group had RLU ratios >5; this is comparable to the values obtained following live ChPV inoculation (unpublished data).

It is also interesting to note that although a single vaccination containing only 5 μg plasmid DNA was

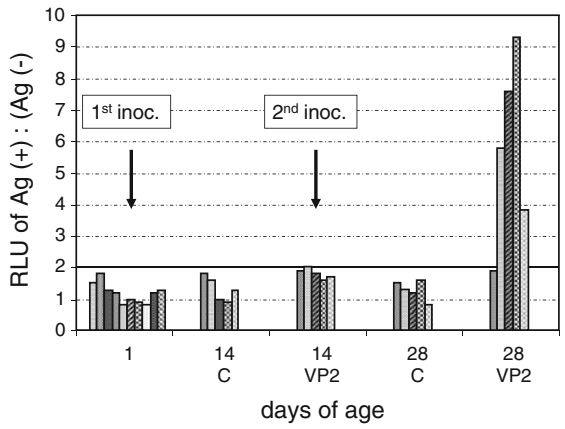

Fig. 4 ELISA antibody titer of chickens following immunization with bicistronic vector expressing codon-optimized ChPV VP2. Readings are expressed as the ratio of RLU obtained for the positive antigen divided by RLU values for the negative antigen as described in the "Materials and Methods" section

insufficient to elicit a humoral immune response, a boost of only an additional 5 μg elicited humoral immunity in 80 % of the chickens. DNA inoculums between 150 and 400 μg have been reported for chickens injected with plasmids expressing VP2 of chicken anemia virus and infectious bursal disease virus [30, 31].

Overall, these studies provided evidence that codon-optimized VP2 can be expressed both transiently in transfected immortal CEFs and in muscle cells of chickens injected with a naked DNA vaccine. In a natural infections, ChPV VP2 is obviously expressed in chicken enterocytes. Our research has indicated that wild-type VP2 sequence is toxic to immortal CEFs (DF-1). The mechanism is unknown but expression of VP2 of other parvoviruses in certain cell types "non-permissive" have been reported for parvovirus B19 [19]. In this report, they demonstrated that VP1 and VP2 expression levels following transient transfection of plasmid were high in CD36+ EPCs, very low in semi-permissive (UT7/Epo-S1) and 293T cells, and undetectable in non-permissive HeLA cells. We postulate that an RNAi mechanism may be responsible for the lack of expression of ChPV VP2 in non-permissive CEFs. In permissive cells such as chicken enterocyte and muscle cells expression is allowed due to the lack of host microRNAs complementary to ChPV RNA or absence of host mRNAs that are the target of dicer-processed parvovirus RNA.

It may be of value to assess whether a live transgenic *E. coli* strain containing the codon-optimized VP2 gene could be used as an oral DNA vaccination for mass vaccination of chickens against RSS. It has recently been reported that oral administration of transgenic *E. coli* containing the VP2 of infectious bursal disease confers protective immune response in chickens [32].

In order to develop a more conventional vaccine, which was the original objective, strategies were designed to rescue the codon-optimized VP2 gene contained in the bicistronic RFP cassette into the thymidine kinase locus of MeHV-1. To accomplish this, a 394 bp oligonucleotide containing restriction endonuclease sites between thymidine kinase flanking sequences necessary for homologous recombination was synthesized commercially and cloned into a donor plasmid. Two donor plasmids containing the codon-optimized VP2 gene within the bicistronic RFP cassette VP2 gene in either orientation (sense and anti-sense) relative to the transcription of the TK gene in the MeHV-1 genome were isolated and used in co-transfection experiments. As shown in Fig. 5, MeHV-1 plaques expressing the downstream RFP were only detected in cells transfected with the plasmid containing the VP2 gene in the

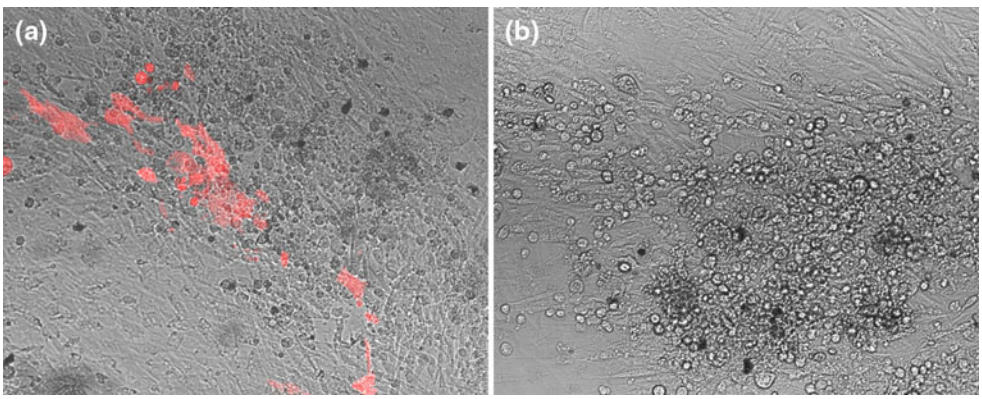

Fig. 5 Expression of ChPV VP2 protein in CEFs infected with recombinant meleagrid herpesvirus-1 as detected by immunocytochemical staining 36 h post-infection. Merged light and fluorescent microscope images of infected CEFs with recombinant meleagrid herpesvirus-1 containing the VP2 gene in the sense orientation (**a**) and the recombinant containing the VP2 gene in the anti-sense orientation (**b**)

sense orientation. These experiments demonstrated that expression from the cytomegalovirus (CMV) immediate early promoter of a codon-optimized ChPV VP2 gene within the meleagrid herpesvirus genome can occur. The ChPV VP2 protein does not appear to affect MeHV's ability to infect CEFs since meleagrid herpesvirus VP2 recombinants have been plaque purified (3×). Pathogenicity studies in chickens vaccinated with plaque-purified MeHV-1 recombinants expressing codon-optimized VP2 are currently in progress.

Conclusions

In this study, the capsid protein VP2 of ChPV was expressed in CEFs only after transfection of plasmid containing codon-optimized VP2. Expression in CEFs using a bicistronic vector containing the wild-type (non-codon-optimized) VP2 gene upstream of an IRES-RFP reporter gene indicated toxicity at the level of RNA. Chickens vaccinated with plasmid constructs containing codon-optimized VP2 generated humoral immunity as measure by ELISA. A recombinant meleagrid herpesvirus containing the codon-optimized VP2 of ChPV was constructed and should be useful as a vaccine against RSS of chickens.

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